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Opinion

Rethinking Thymic Tolerance: Lessons from Mice

Sarah Inglesfield,¹ Emilie J. Cosway,¹ William E. Jenkinson,¹ and Graham Anderson^{1,*}

In the thymus, distinct cortex and medulla areas emphasize the division of labor in selection events shaping the $\alpha\beta$ T cell receptor repertoire. For example, MHC restriction via positive selection is a unique property of epithelial cells in the thymic cortex. Far less clear are the events controlling tolerance induction in the medulla. By acting in concert through multiple roles, including antigen production/presentation and chemokine-mediated control of migration, we propose that medullary epithelium and dendritic cells collectively enable the medulla to balance T cell production with negative selection and Foxp3⁺ regulatory T cell (Treg) development. We examine here the features of these medullary resident cells and their roles in T cell tolerance, and discuss how imbalance in the thymus can result in loss of T cell tolerance.

Thymic Medulla and the Control of T Cell Tolerance

The thymus produces multiple T cell types that play key roles in both innate and adaptive immune responses. The importance of these responses has now been shown in sister lineages of vertebrates, long after the function of the thymus was proven for jawed vertebrates in the 1960s [1,2]. Indeed, a key feature of adaptive immunity lies in its ability to generate a wide diversity of antigen receptors that target non-self. For T cells, this is achieved by T cell receptor (TCR) gene rearrangements that take place during T cell development in the thymus. Because of the random nature of gene rearrangement, the thymus imposes stringent mechanisms that shape the $\alpha\beta$ TCR repertoire. Such processes are crucial because they ensure that **$\alpha\beta$ T cells** (see [Glossary](#)) leaving the thymus are not only biased towards the recognition of self-MHC proteins but also tolerant to self-antigens. To achieve this the thymus creates a division of labor: the cortex imposes MHC restriction via positive selection, while the medulla imposes T cell tolerance via a combination of negative selection and Foxp3⁺ Treg development.

The generation of $\alpha\beta$ T cells in the thymus involves immature thymocytes being subjected to sequential checkpoints as they undergo intrathymic migration. To ensure that the thymus produces $\alpha\beta$ T cells capable of antigen recognition in peripheral tissues, the cortex supports positive selection of immature CD4⁺CD8⁺ double-positive (DP) thymocytes that recognize self-peptide/MHC complexes produced and expressed by cortical thymic epithelial cells (cTECs). This process rescues DP thymocytes from cell death and triggers further differentiation, including expression of the chemokine receptor CCR7 that guides newly selected thymocytes into the medulla [3,4].

Because **positive selection** results in the survival of thymocytes that recognize self-peptide/MHC, additional selection mechanisms ensure that T cell development produces functional thymocytes that are tolerant to self-antigens. The thymic medulla provides a specialized micro-environment to support these events, and **medullary thymic epithelial cells** (mTECs) and **dendritic cells** (DCs) combine to enforce two key processes that are essential for tolerance. First,

Highlights

Foxp3⁺ Treg development and negative selection in the thymus remain key components of T cell tolerance mechanisms, both of which are controlled by the thymic medulla.

Medulla dysgenesis does not necessarily predispose to a loss of T cell tolerance, arguing that thymic medulla function can be preserved despite limitations in the availability of medullary thymic epithelial cells (mTECs).

Recently identified heterogeneity within the thymic stroma suggests that multiple subsets of mTECs play active roles in imposing T cell tolerance.

Accurate examination of the rates and mechanisms controlling Foxp3⁺ Treg selection in the thymus must take into account the presence of peripheral recirculating cells within the Foxp3⁺ intrathymic Treg pool.

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CD4⁺ and CD8⁺ single-positive (SP) thymocytes undergo **negative selection** if they express $\alpha\beta$ TCRs capable of high-affinity recognition of self-antigens in the medulla [5]. Thus, **clonal deletion** is an effective means to eliminate at least some self-reactive thymocytes. Related to this, at least for CD8⁺ $\alpha\beta$ T cells in humans, **thymic tolerance** mechanisms do not lead to the removal of all self-reactive thymocytes, but effectively ‘prune’ the frequency of autoreactive cells that leave the thymus, a process that may avoid the presence of ‘holes’ in the $\alpha\beta$ TCR repertoire [6]. To compensate for potential inefficiencies in negative selection, the medulla also subverts a fraction of CD4⁺ SP thymocytes into the **Foxp3⁺ regulatory T cell** (Treg) lineage [7]. When exported from the thymus, these cells are highly effective at limiting autoreactive responses initiated by T cells that escape thymic tolerance [8]. As with negative selection in mouse thymus, both mTECs and thymic DCs are key regulators of Foxp3⁺ Treg development [9,10]. However, although the importance of the medulla is clear, how this site is specialized to impart these crucial functions remains unanswered. This may be partly due to the complex heterogeneity in all the key cellular players (mTECs, DCs, Tregs), and which must be unraveled to gain a better understanding of events controlling thymic tolerance. Unraveling this complexity will not only give essential insight into how a specialized lymphoid microenvironment is controlled but we also expect that this will be important in the characterization and treatment of autoimmune diseases.

New Complexity in Medullary Thymic Epithelium

The ability of the medulla to guide maturation of diverse T cell subsets, including conventional SP thymocytes, as well as CD4⁺Foxp3⁺ Treg and **invariant natural killer T cell (iNKT) subsets**, is mirrored by a high degree of mTEC heterogeneity [11]. Although immunohistochemistry and flow cytometry findings have well discriminated cTECs from mTECs in the murine thymus, the extent of mTEC heterogeneity has only recently begun to be appreciated. In the steady-state adult murine thymus, mTECs can be broadly subdivided into two distinguishable MHC-II^{low}CD80^{low} (mTEC^{lo}) and MHC-II^{high}CD80^{high} (mTEC^{hi}) subsets. Their relationship has been proposed to involve a linear developmental sequence, where mTEC^{lo} cells can act as precursors for mature mTEC^{hi} cells [12]. Of note, the transition of mTEC^{lo} to mTEC^{hi} can be driven via multiple TNF receptor superfamily (TNFRSF) signals, including RANK and CD40, and involves the upregulation of the **autoimmune regulator** (Aire), as well as the expression of diverse **peripheral tissue antigens** (PTAs) such as insulin 2 and salivary protein 1 [12–16]. The direct consequence of intrathymic loss of single Aire-dependent PTA expression for T cell tolerance is demonstrated by the manifestation of autoimmune eye disease in Aire-deficient mice that lack intrathymic expression of the eye-associated interphotoreceptor retinoid-binding protein (IRBP) [17]. In addition to the role of Aire in the regulation of PTA expression by mTEC^{hi} [13], the transcription factor Fezf2 has also been suggested to act as an additional regulator of distinct panels of PTAs in mTECs [18]. In support, mTECs isolated from Fezf2-deficient mice exhibited an Aire-independent reduction in expression of PTAs – such as the testis-associated antigen fatty acid-binding protein 9 – compared to wild-type mice, generating thymocytes with autoimmune potential [18]. Indeed, although Aire expression was restricted to mTEC^{hi} cells, Fezf2 was reported not only in mTEC^{hi} but also in mTEC^{lo} cells; moreover, the development of Fezf2⁺ mTECs was thought to be controlled via **lymphotoxin β receptor** (LT β R)-dependent signaling [18]. However, recent studies using murine models [19] have shown that both Aire and Fezf2 are expressed by mTECs in LT β R-deficient mice, and that the development of Aire⁺ and Fezf2⁺ mTEC can be driven by stimulation of RANK in *in vitro* thymic organ cultures, a finding consistent with the ability of RANK to drive mTEC^{hi} development from mTEC^{lo} precursors [12].

In earlier studies, detection by flow cytometry of accumulating mTEC^{lo} cells in thymuses from mice of increasing age raised an interesting possibility: either mTEC precursors accumulate with age owing to a bottleneck in developmental transition, or the mTEC^{lo} subset itself included functionally

Glossary

$\alpha\beta$ T cells: express the $\alpha\beta$ form of the T cell receptor (TCR) and recognize peptide fragments presented by major histocompatibility complex (MHC) proteins.

Autoimmune regulator (Aire): the *Aire* gene is expressed by medullary thymic epithelial cells (mTECs) and contributes to controlling self-antigen expression.

***Batf3*^{−/−} mice:** mutant mice that lack exons 1–2 of the gene encoding basic leucine-zipper transcription factor. This mutation tends to target CD11c⁺ conventional DC1 (cDC1) cells more than any other immune cell because cDC1 cells express high levels of *Batf3*. This leads to their absence from *Batf3*^{−/−} mice.

Central Tregs (cTregs): a Treg subset defined by expression of CD62L and lack of CD44. They are believed to originate within the thymus as part of *de novo* regulatory T cell (Treg) development. cTregs circulate through secondary lymphoid tissue and are believed to control T cell priming.

Clonal deletion: the elimination via apoptosis of self-reactive thymocytes in the thymus.

Cross-presentation: the acquisition of self-antigen via trogocytosis or apoptotic release by thymic dendritic cell subsets from Aire-expressing mTECs.

CytoF: a mass cytometry technique that uses metal-conjugated antibodies to enable detailed phenotyping of cells.

Dendritic cells (DCs): a heterogeneous mixture of intra- and extra-thymically generated antigen-presenting cells that reside predominantly in medullary areas and mediate thymic tolerance mechanisms through self-antigen presentation.

Effector Tregs (eTregs): a Treg subset defined by expression of CD44 and lack of CD62L. Believed to result from differentiation of cTregs within peripheral tissue. Effector Tregs predominate in non-lymphoid tissue and control T cell effector functions in part via IL-10 secretion.

Epidermal cornification: a terminal differentiation program of keratinocytes in the skin.

Foxp3⁺ regulatory T cells (Tregs): a subset of CD4⁺ $\alpha\beta$ T cells, defined

mature cells rather than solely acting as a reservoir of immature mTECs [20]. In support of the latter, expression of terminal differentiation markers associated with **epidermal cornification**, such as cytokeratin 10, has been described in mTEC^{lo} cells [21]. Moreover chemokine CCL21 has been shown to be predominantly expressed by a subset of mTEC^{lo} cells in murine thymus via flow cytometry [3,22]; this is relevant because CCL21 is essential for positively attracting selected CCR7⁺ SP thymocytes into medullary regions, thereby ensuring that developing thymocytes are exposed to PTA-expressing mTECs for central tolerance induction [3,22]. Thus, the mTEC^{lo} subset not only contains immature precursors but also contains functionally mature and specialized subtypes. It remains to be determined how functionally mature CCL21⁺ mTEC^{lo} cells overlap with immature cells capable of giving rise to mature mTEC^{hi} cells via TNFRSF triggering.

Recently, to better understand mTEC heterogeneity, two complementary studies closely examined mTEC diversity. Using a murine tamoxifen-inducible Aire-driven fluorescent fate-mapping system, one report identified four mTEC subsets; mTEC^{lo} fractions included cells that had not expressed Aire (defined as pre-Aire), as well as a fate-mapped post-Aire fraction, providing evidence for developmental heterogeneity in mTEC^{lo} compartments [21]. Bulk RNA sequencing of these mTEC subsets identified via Aire-expression fate-mapping revealed that post-Aire cells contained transcriptional signatures of cornified epithelium such as expression of involucrin [21]. However, a second signature in the post-Aire mTEC^{lo} fraction correlated with functionally specialized epithelial cells bearing striking hallmarks normally attributed to a specialized epithelial subset, termed **tuft cells**, previously described in peripheral mucosal tissues [21]. Tuft cells consist of mucosal epithelia-associated chemosensory cells that initiate IL-25 production and immune responses to parasites, including helminths [23–25]. Nevertheless, mTECs bearing a tuft cell signature were also found in pre-Aire subsets, and, although some thymic tuft cells passed through an Aire-expressing stage, some tuft cells appeared to develop via Aire-independent routes, suggesting that thymic tuft cells might develop through at least two independent developmental pathways [21]. Whether such differences in maturational pathways correlate with differing functionality remains an open question. In a second, parallel study, unbiased single-cell RNA sequencing of murine mTECs revealed four distinct subsets, termed mTECs I–IV [26]. Although mTEC subsets I–III displayed transcriptional signatures correlating with immature progenitors, subset IV mature mTECs – including Aire⁺ cells and terminally differentiated post-Aire cells – again provided a tuft cell-like signature. Although previous studies identified cells with molecular characteristics of chemosensory tuft cells in the thymus [27–29], the functional significance of these remains unclear. In this regard, the studies described above [21,26] suggest a functional significance of murine thymic tuft cells in the regulation of intrathymic **type 2 innate lymphoid cells** (ILC2s) and iNKT2 cells, where diphtheria toxin-mediated ablation of post-Aire tuft cells in mice expressing the diphtheria toxin receptor under the control of the Aire promoter led to reduced intrathymic iNKT2 numbers relative to controls; moreover, the absence of Pou2f3-dependent tuft cells in the thymus of Pou2f3-deficient mice resulted in increased numbers of ILC2 cells relative to controls [21,26]. In these reports the chemosensory profile of thymic tuft cells differed from that of their peripheral counterparts; for instance, in contrast to small intestine (SI)-associated tuft cells, thymic tuft cells expressed the taste signal pathway-associated molecule Gnat3, as well as TAS2R family members, suggesting unique chemosensory and functional properties [21]. This finding is further highlighted by the observation that, compared to their intestinal counterparts, thymic tuft cells express MHC class II and can impose central tolerance against tuft cell-associated IL-25 self-antigen [21].

Regulation of Intrathymic DCs

The murine thymic DC pool consists of multiple populations that are distinct in terms of their developmental origins, and probably also in their functional contributions to T cell tolerance

by expression of the transcription factor Foxp3, that control self-reactive immune responses.

Fetal thymus organ culture: *in vitro* system in which embryonic thymus lobes explanted in culture can be used to assess T cell development.

Invariant natural killer T cell (iNKT) subsets: specialized subsets of invariant $\alpha\beta$ T cells produced in the thymus by recognition of CD1d rather than MHC.

Lymphotoxin β receptor (LT β R): a member of the TNF receptor superfamily (TNFRSF3) that is expressed in thymic stromal cells; of key importance in mTEC development.

Macrophage and dendritic cell precursors (MDPs): a bone marrow-restricted progenitor that gives rise to both the monocytoid lineage and common DC progenitors (CDPs).

Medullary thymic epithelial cells (mTECs): form and reside within medullary areas; there are multiple mTEC subsets that are specialized for T cell tolerance and post-selection thymocyte development.

Negative selection: removal of immature thymocytes expressing self-reactive $\alpha\beta$ T cell receptors.

Osteoprotegerin (OPG)-deficient mice: these mice lack the TNF receptor family member TNFRSF11b, which results in a larger thymus medulla.

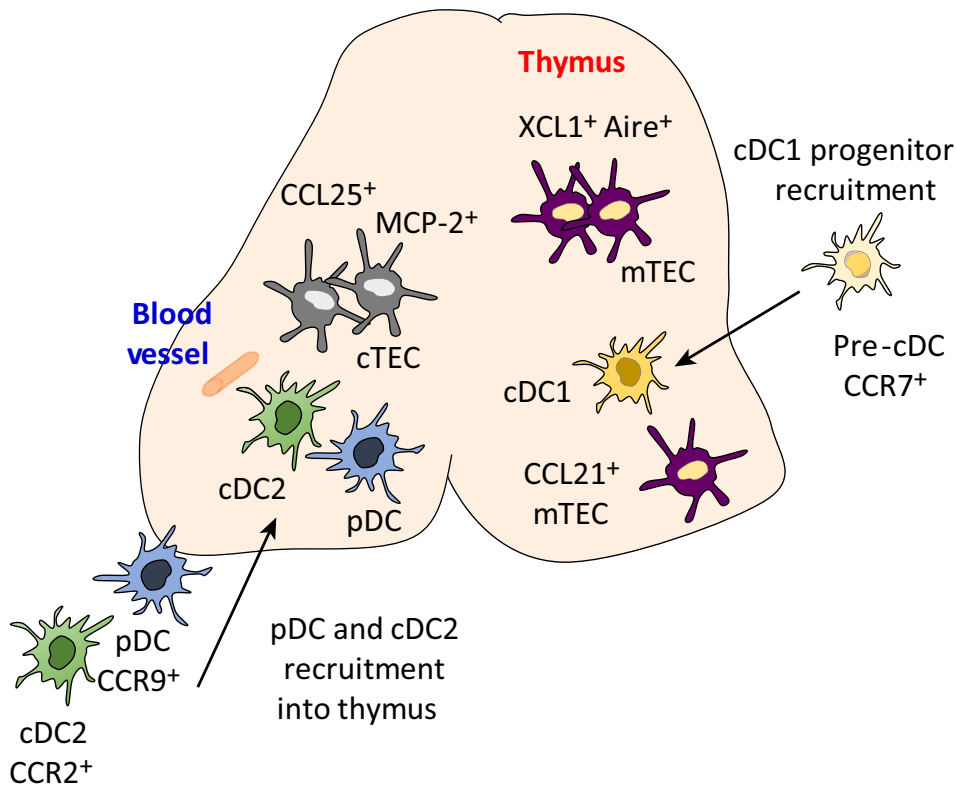
Peripheral tissue antigens: self-antigens expressed in the thymus by mTECs.

Plasmacytoid DCs (pDCs): a subset of DCs that develop within the bone marrow directly from the common DC progenitor. They are CD11c⁺PDCA-1⁺.

Positive selection: an intrathymic process by which CD4⁺CD8⁺ thymocytes expressing $\alpha\beta$ TCRs recognizing MHC are triggered for further maturation.

Rag2pGFP mice: these express the fluorescent protein GFP under the control of the *Rag2* gene promoter (Rag2p); useful tool to measure thymocyte 'age' and distinguish newly produced (GFP⁺) cells from older (GFP[−]) counterparts.

Thymic conventional DCs (cDCs): a DC subset arising from pre-cDCs that develop within the bone marrow



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Figure 1. Heterogeneity in the Murine Intrathymic Dendritic Cell (DC) Pool. The adult murine thymus contains a heterogeneous mixture of DCs that collectively form the intrathymic DC pool. Both plasmacytoid DCs (pDCs) and conventional DC2 (cDC2) cells enter the thymus as mature DCs from peripheral tissues. Their attraction to the thymus involves CCR9 and CCR2, respectively, with chemokine ligands CCL25 and CCL8 being products of thymic stroma. By contrast, conventional DC1 (cDC1) cells are generated intrathymically from immature progenitors (pre-cDCs). Such cells express the chemokine receptor CCR7 and are recruited to the thymus via CCL21 production from medullary thymic epithelial cells (mTECs). Intrathymic DCs are also influenced by mTECs which occurs via Aire-dependent production of the chemokine XCL1.

(Figure 1). For example, **thymic conventional DCs** (cDCs) can be subdivided into $CD8\alpha^+Sirp\alpha^-CD11b^-$ cDC1 and $CD8\alpha^-Sirp\alpha^+CD11b^-$ cDC2 cells. The thymus also contains **plasmacytoid DCs** (pDCs) that are defined by expression of PDCA-1. The mechanisms controlling the formation of the adult intrathymic DC pool are still poorly understood. However, the thymus lacks both **macrophage and DC precursors** (MDPs) and common DC progenitors (CDPs). Regarding the latter, CDPs can give rise to pre-cDCs [30] that exit the bone marrow and enter the periphery where they develop into cDC1 and cDC2 cells [31]. For thymic cDC2 and pDC cells, several studies have shown that these cells migrate into the thymus as mature DCs. For example, in a variety of tracking approaches, including GFP–OVA-loaded DCs into DO11.10 TCR transgenic mice and fluorescent dextran bead/fluosphere injections, injected trackers are endocytosed by DC subsets and are subsequently used to document the appearance of labeled DCs in the thymus from the periphery [32–34]. Furthermore, using blocking monoclonal antibodies, cell adhesion molecules such as endothelial P-selectin and VCAM-1 were shown to be necessary for the thymic entry of pDC and cDC2 cells while also highlighting their migratory nature [35]. Further investigation of $Ccr2^{-/-}$ mice indicated that cDC2 cells are recruited to the thymus via expression of CCR2 and TEC CCL8 expression [33];

and egress into the periphery before developing into either Sirp α -expressing cDCs (cDC2 cells) or Sirp α -lacking cDCs (cDC1 cells). All cDCs have a $CD11c^+PDCA-1^-$ phenotype.

Recirculating thymic Tregs: mature cells that re-enter the thymus from peripheral tissues.

Thymic slices: *in vitro* system where slices of neonatal thymus lobes are explanted in culture to study T cell development.

Thymic tolerance: mechanisms of negative selection and Foxp3 $^+$ Treg development: in combination they help to prevent T cell-mediated autoimmunity.

Traf6 Δ TEC mice: TNF receptor-associated factor-6 (Traf6) is part of a family of adaptor proteins that mediate signaling pathways that can ultimately activate transcription factors such as NF- κ B. Traf6 is specifically deleted on TECs via the $Traf6^{fl/w} \times Foxn1^{Cre}$ mouse cross: this absence leads to depletion of mTECs.

Tuft cells: specialized chemosensory epithelial cells that are found in tissues such as the intestinal mucosa; implicated in the initiation of type 2 immune responses.

Type 2 innate lymphoid cells (ILC2s): innate counterparts of CD4 $^+$ T helper 2 (T_H2) cells that produce type 2 cytokines and coordinate immune responses at barrier sites.

in addition, immunohistochemistry of wild-type and *Ccr2*^{-/-} mice clearly indicated that cDC2 cells localize around blood vessels to capture soluble antigen [33]. For pDCs, CCR9 expression allows access to the gut and also enables their entry to the thymus, where CCL25 is expressed by TECs, as shown in *Ccr9*^{-/-} mouse models in both steady-state and chimeric settings [34]. Considering this, a recent study [36] reported a reduced ability of newborn non-obese diabetic (NOD) mice to support peptide-induced thymocyte deletion compared to 4 week old mice; this impairment in thymocyte deletion correlated with thymic DC alterations, in particular for cDC2 cells [36]. Thus, balance between differing thymic DC subsets may be necessary for optimal tolerance during thymocyte development.

Unlike cDC2 cells and pDCs, thymic cDC1 cells develop within the thymus from migrant precursors. A recent study took advantage of single-cell mRNA sequencing and suggested that pre-cDCs could be subdivided based on SiglecH and Ly6C expression to identify cells primed towards a cDC1/cDC2 lineage [37]. Further, our group utilized various knockout mouse models (*Ccr7*^{-/-}; paucity of lymph node T cell, *plt/plt*; and *Ccl21*^{-/-}) to demonstrate that the CCR7–CCL21a axis is pivotal in pre-cDC recruitment from the bone marrow to the thymus, as well for cDC1 cell maintenance in the thymus (Figure 1) [38]. Once in the thymus, pre-cDCs can develop into cDC1 cells, becoming increasingly activated with enhanced MHCII and CD80/CD86 expression (as previously suggested in the literature through adoptive transfer experiments and phenotypic analysis); the activation of these cDC1 cells is likely further linked to their ability to express relevant $\alpha\beta$ TCR ligands and costimulatory molecules for thymocyte selection [31,38]. However, this clearly warrants further investigation. Although the above findings indicate current knowledge on the mechanisms that control the thymic recruitment and development of murine DC, little is known about what controls intrathymic DC positioning. Indeed, it is still unclear where in medullary areas cDC1, cDC2, and pDC cells reside. However, one study showed expression of the chemokine receptor XCR1 by thymic cDC1 cells; moreover, using RT-PCR analysis of wild-type and Aire-deficient mice, the intrathymic expression of the XCR1 ligand XCL1 was shown to be controlled by mTEC expression of Aire [39]. Thus, in both Aire-deficient and XCL1-deficient mice, intrathymic positioning of DCs was subsequently altered, with a reduced ability to reside in medulla areas relative to DCs from wild-type mice (as evidenced from immunofluorescence). This DC mispositioning is accompanied by defective self-tolerance and altered induction of Treg populations, leading to autoimmune infiltrates in nude mice transfer models [39]. This suggests that mTECs may have the ability to further influence tolerance mechanisms by ensuring that thymic DCs migrate to relevant medullary areas. Finally, although these studies demonstrate a requirement for multiple cellular interactions during thymic tolerance, there is a lack of systems that would permit careful analysis of individual cell–cell interactions controlling this process. As such, and as recently shown for TECs [40], the development of new fluorescent reporter tools that enable tracking/visualization of distinct TEC/DC/thymocyte subsets will no doubt aid further studies in this area.

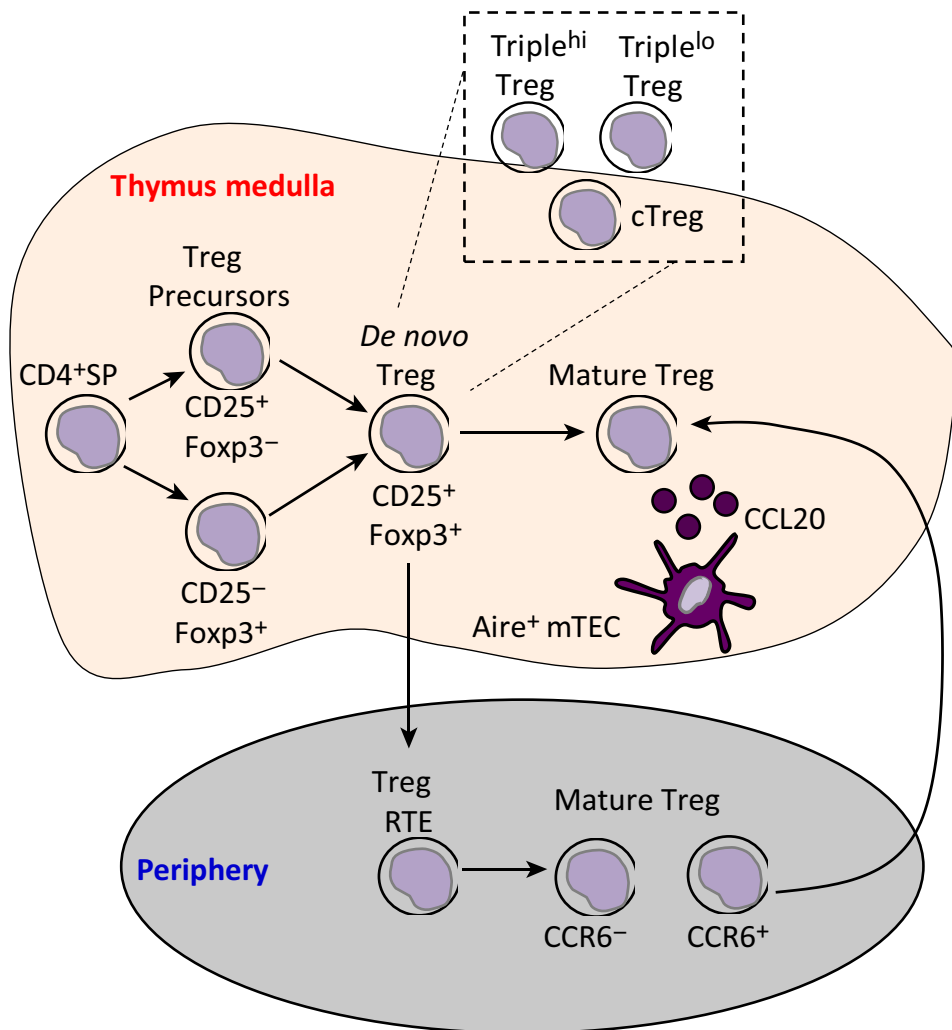
Further evidence of the importance of thymic DC for T cell tolerance comes from studies in which the key regulator of thymic medulla development, lymphotoxin β receptor (LT β R), was deleted from TEC. This was possible by crossing *Foxn1*^{Cre} mice with *Ltbr*^{fl/fl} mice to generate *Foxn1*^{Cre}*Ltbr*^{fl/fl} (LT β R^{TEC} mice) which have a specific deletion of LT β R in Foxn1-expressing cells, in this instance allowing specific targeting of LT β R in all TECs [41]. However, when these mice were compared to germline *Ltbr*^{-/-} mice it became apparent that previously described autoimmunity in these germline mice was not simply due to the reported disrupted thymic medulla [41]. Instead, through such comparative analysis, loss of tolerance mapped to alterations in mouse thymic DCs in the germline *Ltbr*^{-/-} mice, which in turn limited negative selection – as evidenced from reduced numbers of thymocytes deemed to undergo negative

selection ($CD5^+CD69^+$ caspase-3⁺) relative to controls [19]. Loss of mTECs alone with no DC alterations, as seen in $LT\beta R^{TEC}$ mice, did not result in failed thymic tolerance and a lack of induction of autoimmune infiltrates or autoantibodies in tissue and serum analyses. Compared to autoimmune germline $Ltbr^{-/-}$ mice with both mTEC and DC disruption, this suggests that DC availability, and not mTEC availability, is crucial for the ability of the medulla to impose negative selection. However, another study used other murine models of mTEC deficiency (***Traf6*ΔTEC mice**), $CD8\alpha^+$ cDC1 deficiency (***Batf3*^{-/-} mice**), or $Batf3^{-/-}$ *Traf6*ΔTEC double-knockout mice to investigate, in comparison to wild-type mice, the interplay between these two thymic populations during tolerance induction. This study suggested that loss of both mTECs and cDC1 cells was required for loss of thymic tolerance, highlighting the ability of mTECs and DCs to partially compensate for one another to essentially prevent overt autoimmunity [42]. In line with this, investigations were made using autoimmune lymphoplasia *aly/aly* mice that harbor a point mutation in the NF-κB-inducing kinase (NIK) gene and suffer from absence of peripheral lymph nodes and Peyer's patches. Using *aly/aly* adult mice as recipients that were lethally irradiated and reconstituted with wild-type bone marrow, mTECs and cDC1 cells were numerically reduced compared to similarly treated *aly/+* irradiated host mice [43]. Crossing of *aly/+* or *aly/aly* mice with RIP-OVA transgenic mice – with OVA expression as a model antigen in mTEC – suggested defects in negative selection of TCR transgenic thymocytes [43]. Although further studies are needed to examine how interactions between DC and mTEC influence tolerance induction, it is becoming apparent that interactions between cDC1 cells and mTECs may be necessary to drive antigen presentation/**cross-presentation** during central tolerance; this may entail Aire-expressing mTECs as the main providers of self-antigen, with DCs enabling further opportunities for self-antigen expression within the thymus, thus coordinating appropriate screening of developing thymocytes, as well as the effective removal of autoreactive thymocytes.

Complexity of the Intrathymic Treg Population

Developmental Stages in Treg Development

Despite the known importance of the medulla for Foxp3⁺ Treg cell development, the process of initial commitment to the thymic Treg lineage remains controversial. Indeed, two distinct subsets of $CD4^+$ SP thymocytes have been reported to be direct precursors of mouse $CD25^+Foxp3^+$ Tregs (Figure 2) [44,45]. Thus, both $CD25^+Foxp3^-$ [44] and $CD25^-Foxp3^+$ [45] $CD4^+$ SP thymocytes were shown to give rise to $CD25^+Foxp3^+$ Treg following their intrathymic injection [44] or adoptive cell transfer into adult mice [45], respectively; this indicated that both populations could act as intrathymic Treg precursors. However, their relative contributions to the newly produced intrathymic Treg pool remains unclear. Indeed, why Treg development might involve two separate developmental pathways involving distinct precursor pools is not known. Consequently, experimental systems which give clear insight into the precursor–product relationships in Treg development are required. Accordingly, recent work in Nr4a3–Tocky mice (which report TCR signaling dynamics by virtue of a fluorescent timer protein) has offered novel insights into the dynamics of Treg development [46]. In these mice, TCR signaling induces an unstable chromophore that transiently emits blue fluorescence before maturing to a stable chromophore that emits red fluorescence. Thus, when cells first receive a TCR trigger they are blue⁺red⁻. Over time, such cells then transition to a blue⁺red⁺ stage and, without persistent TCR triggering, become blue⁻red⁺. In this recent study, $CD25^+Foxp3^-$ $CD4^+$ SP thymocytes were shown to be predominantly blue⁺red⁻, whereas both $CD25^+Foxp3^+$ Tregs and $CD25^-Foxp3^+$ thymocytes were predominantly blue⁺red⁺. This suggested that $CD25^+Foxp3^-$ thymocytes could receive a TCR signal, transitioning to the $CD25^+Foxp3^+$ stage, and supporting the idea that at least some Treg precursors reside within the $CD25^+Foxp3^-$ subset [46]. By contrast, in the same system, $CD25^-Foxp3^+$ thymocytes



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Figure 2. Foxp3⁺ Regulatory T Cell (Treg) Development and Recirculation in the Murine Thymus. The thymic medulla supports the commitment and development of Foxp3⁺ Tregs from newly selected CD4⁺ single-positive (SP) thymocytes. Two distinct Treg precursor subsets (CD25⁺Foxp3⁻ and CD25⁻Foxp3⁺) have been reported to give rise to *de novo* CD25⁺Foxp3⁺ Tregs. Newly produced Tregs are probably a heterogeneous population, containing Triple^{hi} (CD25^{hi}PD1^{hi}GITR^{hi}), Triple^{lo} (CD25^{lo}PD1^{lo}GITR^{lo}), and central Tregs (cTregs; CD62L⁺CD44⁻). *De novo* Tregs that leave the thymus as recent thymic emigrants (RTEs) then join the mature peripheral pool. Mature peripheral CCR6⁺ Tregs can also re-enter the thymus in response to CCL20 production from Aire⁺ medullary thymic epithelial cells (mTECs). In addition, *de novo* produced Tregs may also be retained in the thymus and persist as thymus-resident cells.

were shown to be in a similar stage of development into Tregs, at least in terms of timing of TCR signaling, based on both populations predominantly being blue⁺red⁺, and indicating persistent TCR signaling [46]. This implied that at least some Foxp3⁺CD25⁻ precursors receive a TCR signal but do not immediately differentiate into CD25⁺Foxp3⁺ Tregs; instead they may remain as Foxp3⁺CD25⁻ thymocytes for an undefined time-period [46]. However, work in **Rag2GFP mice** still suggests a precursor role for these cells. Specifically, in these mice GFP is produced when the recombinase activation gene 2 (*Rag2*) locus is active; following cessation of *Rag2* gene expression after positive selection, accumulated GFP undergoes exponential decay over

approximately 3 weeks *in vivo*, until cells become GFP⁺ [47,48]. Thus, newly produced thymocytes undergoing intrathymic development can be identified as GFP⁺, whereas mature T cells are GFP⁺. In addition, the expression of GFP corresponds to cell age, such that the younger the cell, the higher the level of GFP expression is. Notably, in Rag2GFP mice the majority of CD25⁺Foxp3⁺CD4⁺ SP thymocytes have been identified as GFP⁺, suggesting that these cells might belong to a phase of recent intrathymic T cell development [49]. In sum, the combined evidence supports the hypothesis of distinct populations of Treg precursors in the mouse thymus. It will be important to determine whether Treg precursor heterogeneity explains the reported functional heterogeneity in thymically produced Tregs (discussed below), or if it is explained by the stochastic acquisition of Foxp3 and CD25 expression during Treg development.

Heterogeneity in *De Novo* Intrathymic Treg Cell Production

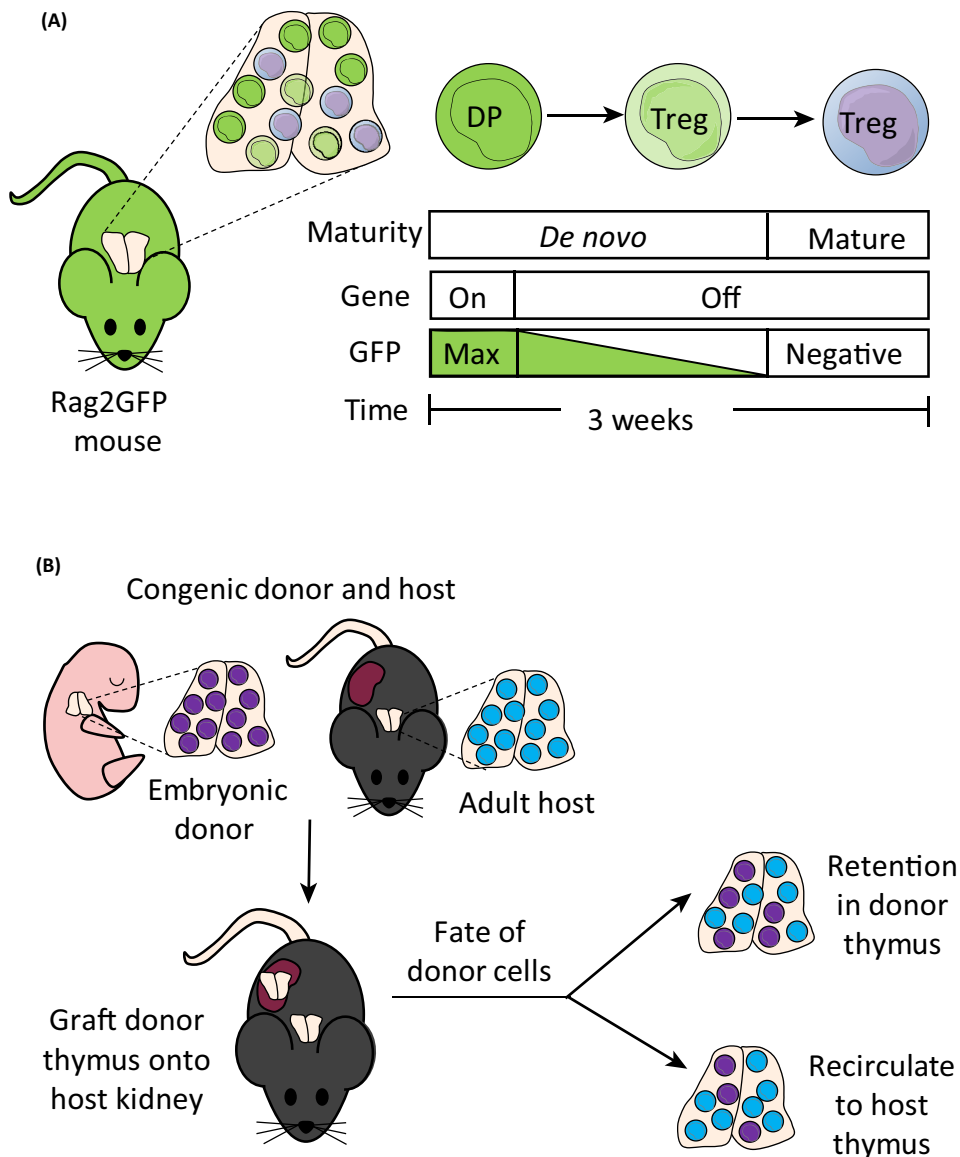
The presence of two intrathymic populations with Treg precursor potential may be linked to the ability of the thymus to produce multiple Treg subsets that are phenotypically and functionally distinct. Indeed, peripheral Tregs display both phenotypic and functional heterogeneity, including the expression of adhesion molecules and chemokine receptors [50,51], which likely underpins their ability to control diverse immune responses across disparate body sites. However, whether this diversity is established during T cell development or occurs following thymic export, is unclear. Relevant to this, two Foxp3⁺ Treg types have been identified in adult mouse spleen [50]: **central Tregs** (cTregs, CD44⁺CD62L⁺) and **effector Tregs** (eTregs, CD44⁺CD62L⁺). By contrast, in Rag2GFP mice, newly produced thymic Treg have been reported to all be cTregs, suggesting that T cell development might produce cTregs, that subsequently exit the thymus and convert into more mature eTregs in peripheral tissues [51]. This is supported by adoptive transfers of flow cytometry-sorted cTregs in mice, which were shown to acquire a CD44^{hi}CD62L^{lo} eTreg phenotype following transfer [50]. By contrast, other work identified ‘Triple^{hi}’ [CD25^{hi}, programmed cell death protein 1 (PD1)^{hi}, glucocorticoid-Induced TNFR-related protein (GITR)^{hi}] and ‘Triple^{lo}’ (CD25^{lo}PD1^{lo}GITR^{lo}) subsets of intrathymic Tregs in mice, supporting the idea that multiple distinct Treg types are generated in the thymus [51] (Figure 2). Furthermore, both subsets consisted of newly produced GFP⁺ thymocytes in Rag2GFP mice which, as previously discussed, provide a so-called ‘timer’ for T cell development. Because both subsets contain GFP⁺ thymocytes, and both subsets seemed to be a direct result of new development, this rules out the possibility that the heterogeneity might solely be explained by one subset representing mature peripheral GFP⁺ **recirculating thymic Tregs** [51]. Moreover, this report [51] also showed that the sequenced TCR repertoire of sorted Triple^{hi} and Triple^{lo} Tregs differed and correlated with differing affinity for antigen, given that analysis of TCR signal strength using Nur77–GFP reporter expression showed that Triple^{hi} Tregs had higher Nur77–GFP expression than did Triple^{lo} Tregs, and thus likely received a stronger TCR signal. Finally, by exposing TCR transgenic thymocytes to antigens with different affinities, this study [51] demonstrated that threshold-affinity antigen induced Triple^{lo} Tregs whereas high-affinity antigen induced Triple^{hi} Tregs. Overall, these findings suggested that thymic generation of different Treg subsets might be determined by the affinity of the Treg TCR for self-antigen, with high or low affinity resulting in Triple^{hi} and Triple^{lo} Tregs, respectively [51]. This is an intriguing finding because it hints at the putative underlying mechanisms instructing thymic Treg diversity. Moreover, such findings are significant because they suggest that the thymus can produce multiple Treg types which may also have distinct regulatory properties. Consequently, Treg development in the thymus might operate via multiple pathways, perhaps including differences at the Treg precursor stage, as well as differing requirements for medulla support, although this remains hypothetical. Overall, although we are aware of diversity within the Treg population, the origin of this heterogeneity remains uncertain. With the availability of

advanced immunophenotyping and profiling, including **CyTOF** and single-cell RNA analysis of Treg populations, it should be possible to provide in-depth analysis of the stages of intrathymic Treg development and the signals that control them.

Mature Treg Cells in the Thymus

The presence of distinct subsets within the *de novo* Treg compartment is not the only source of heterogeneity within the thymus. There is also developmental heterogeneity, and mature peripherally derived Tregs are present in the thymus alongside their newly produced counterparts [49,52,53]. This has perhaps been most comprehensively demonstrated using Rag2GFP mice, where thymocytes undergoing continued intrathymic development can be identified as GFP⁺, whereas more mature T cells are GFP[−] [47,48]. In these mice, GFP[−] Tregs were found to heavily contaminate the intrathymic Foxp3⁺CD25⁺ Treg population, becoming increasingly dominant with age [49,52,53]. To interrogate the origin of these mature Tregs, thymus transplantation models and adoptive cell transfers of candidate Treg progenitors have also proved to be useful tools (Figure 3) [49,52,53]. In thymus transplants, embryonic lymphoid thymus lobes are surgically grafted into congenially different hosts, and the fate of the single wave of graft-derived Tregs can then be assessed. Similarly, adoptive transfers use intravenous transfer of congenically marked Tregs that can be followed over time. Consistent with at least some GFP[−] Tregs in the thymus being recirculating cells, the presence of graft-derived or transferred Tregs in the host thymus directly demonstrates thymic recirculation of peripheral Tregs in mice [49,52,53]. However, there are important caveats to both experimental systems. For example, first, it is not fully understood whether the transplantation of embryonic thymus into an adult setting fully recapitulates the cell migration and/or development that take place between adult tissues. Second, experiments involving transfer of non-physiologically large numbers of cells may mean that niche availability within tissues can impact on the ability of transferred cells to reveal their developmental potential because previously filled niches may restrict the developmental potential of the transferred cells. Finally, in addition to thymic recirculation, other studies [52] have also shown in grafted thymus lobes that the proportion of graft-derived Tregs is greater in the grafted thymus than in the host thymus, indicating that the presence of GFP[−] Tregs in the thymus might be explained by their long-term retention. Although the functional significance of this retention is unclear, it is interesting that CD1d-restricted iNKT cells can also reside in the thymus for long periods, where they can influence T cell development, including thymic export [54,55]. However, the relative contributions that thymic recirculation and retention make to modulate the presence of mature Tregs in the thymus remains unclear.

For thymic recirculation, efforts have been made to understand the mechanism controlling this process and also its possible significance to thymus function. Although earlier studies indicated that CXCR4 plays a role in the recruitment of peripheral Tregs to the thymus [53], analysis of *Cd4^{cre}/Cxcr4^{flxed}* mice showed no alterations in the intrathymic Treg pool [56]. Indeed, recent studies have shown that, in *Ccr6^{−/−}* Rag2GFP mice, thymic recirculating Treg numbers are reduced relative to wild-type controls, suggesting that CCR6 is an important regulator of peripheral Treg recruitment to the thymus [57]. Moreover, the medulla may be a key regulator of Treg recirculation, at least for CCR6-mediated thymic recirculation, because the CCR6 ligand CCL20 is detectable in mTECs. Thus, mTECs may generate a CCL20 chemokine gradient that recruits peripheral Tregs to the thymus and allows recirculating Tregs to specifically localize to the medulla [57]. In addition, CCL20 expression in mTEC from *Aire^{−/−}* mice is reduced relative to wild-type mice, and *Aire^{−/−}* Rag2GFP mice also exhibit reduced levels of Rag2GFP[−] recirculating Tregs (relative to wild-type controls); this supports a role for Aire in Treg recirculation to the thymus through mTEC secretion of CCL20 (Figure 2) [57]. That Aire is involved in the



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Figure 3. Approaches to Assessing Developmental Heterogeneity in Murine Intrathymic Regulatory T Cell (Treg) Populations. (A) Rag2GFP mice provide a molecular clock with which to study Treg development. The *Rag2* gene is expressed by immature double-positive (DP) thymocytes during T cell development, and GFP is produced. Expression of *Rag2* then terminates following positive selection. Consequently, no further GFP protein is produced by single-positive (SP) thymocytes, and existing GFP decays exponentially throughout *de novo* Treg development. The GFP signal is lost after approximately 3 weeks, resulting in the presence of mature GFP[−] T cells in peripheral tissues. In the thymus of Rag2GFP mice, Tregs are a mixture of GFP⁺ (*de novo* produced) and GFP[−] (mature recirculating) cells. (B) Thymus transplantation enables mapping of the fate of a single wave of T cell development, including Foxp3⁺ Tregs. Congenic embryonic thymic lobes (donor) are grafted under the kidney capsule of an adult mouse (host), and, at appropriate timepoints, host and donor thymus tissue can be analyzed for the presence of host and graft-derived T cells. The fate of the donor Tregs (purple) is likely a combination of retention in the donor thymus and recirculation to the host thymus.

regulation of a chemokine that can influence Treg migration in the thymus draws parallels with its role in controlling XCL1-mediated positioning of thymic DCs [39].

These findings collectively underline the multiple roles of Aire in regulating not only self-antigen expression but also cellular positioning in the medulla. Why do mature Tregs, that are important enforcers of immune tolerance in the periphery, return to the thymus? As mice grow older, they exhibit a reduction in thymic Treg output and an increased accumulation of mature Tregs within the thymus; this correlation suggests that mature Tregs might potentially act to suppress new Treg development, although this remains speculative and it is unclear what purpose inhibiting new Treg development might serve [53,58]. Nevertheless, *in vitro* experiments support this theory. For instance, the addition of mature Tregs to **fetal thymus organ cultures** and **thymic slices** can impair new Treg development [53,58]. Indeed, one possible mechanism is that mature recirculating Tregs can compete for intrathymic IL-2, thus limiting the availability of this key cytokine for new Treg production [53,58]. Thus, Treg recirculation might be able to dictate the size of the thymic Treg niche. However, other findings might argue against this possibility. For example, in *Aire*^{-/-} mice where recirculating thymic Tregs are reduced relative to wild-type mice, presumably less IL-2 competition would ensue because *de novo* Tregs would have fewer competitors for the cytokine, and *de novo* Treg numbers would increase; however, in *Aire*^{-/-} mice, *de novo* Treg numbers were not increased [57]. Conversely, **osteoprotegerin (OPG)-deficient mice** exhibit increased thymic Treg recirculation relative to wild-type, which potentially provides more IL-2 competition; however, they do not show decreased *de novo* Treg development compared to wild-type control mice [59]. As such, the relationship between mature thymic Tregs and *de novo* Tregs remains unclear, and any functional significance of the presence of recirculating peripheral Tregs in the thymus requires further investigation.

Regardless of any direct functional impact on thymic Treg development, the presence of mature recirculating Tregs in the thymus represents a confounding factor for accurate analysis of Treg development. The importance of this is clear from analysis of T cell development in *Ccr7*^{-/-} mice [49]. Initially these mice offered a perplexing phenotype: despite exhibiting a severely compromised ability of thymocytes to enter the medulla, the numbers of medulla-dependent Foxp3⁺ Tregs were increased relative to wild-type controls [60,61]. Moreover, analysis of *Ccr7*^{-/-}/Rag2GFP mice showed that, although *de novo* development and recent thymic emigrant (RTE) frequency were unaffected, an increase in thymic recirculating Tregs contributed to increased thymic Treg numbers relative to Rag2GFP wild-type controls. Thus, despite normal thymic Treg development, an abnormal phenotype was observed as a result of the impact of CCR7 loss on mature thymic Tregs. This highlights the notion that mature and *de novo* Treg populations need to be considered separately when assessing thymic development. Hence, to provide a clearer understanding of the mechanisms and stages of Treg selection in the thymus, the use of additional experimental systems is required (e.g., Rag2GFP mice); these would allow the exclusion of recirculating cells from the newly produced Treg pool and facilitate direct examination of *de novo* Tregs.

In summary, mature Tregs within the thymus need to be considered when interrogating *de novo* Treg development for two major reasons. First, they are a confounding factor in any investigation on Treg development because they may erroneously increase measurements of new Treg production. Second, mature Tregs themselves may play some role in shaping *de novo* Treg development – an avenue that warrants further investigation. Given this possible role for mature Tregs in the thymus, it is important to understand what signals direct them to be retained within the thymus or to recirculate into it.

Concluding Remarks

Multiple cellular compartments of the medulla act in concert to both delete (negative selection) and subvert (Treg selection) thymocytes to achieve a tolerant state. Despite this, how the mechanisms of development and function of the thymic medulla impart such specialization remains enigmatic. Numerous questions therefore clearly remain (see Outstanding Questions). Crucially, both DCs and mTECs are highly heterogeneous in the thymus. Regarding the latter, it is clear that multiple phenotypically and genotypically distinct subsets exist [21,26]. A key focus of future research must now be the systematic examination of the precursor–product relationships that collectively govern mTEC development. In addition, functional examination of the relative contributions of newly defined mTEC subsets, including their roles in tolerance induction (both negative selection and Treg generation), post-selection thymocyte maturation, and thymic egress, must be performed. In this way, a clearer understanding of the cellular heterogeneity within the thymic medulla will eventually provide an exciting opportunity to manipulate thymic selection mechanisms so as to re-establish the balance of immunity and tolerance that is lost during autoimmunity.

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Outstanding Questions

How does cellular diversity in both mTECs and DCs contribute to the processes of Treg development and negative selection?

What are the relative contributions of negative selection and Treg development to immune tolerance?

Which aspects of medulla development are essential for establishing T cell tolerance, and which are redundant?

How and why do peripheral Foxp3⁺ Tregs home back to the thymus?

From which peripheral tissues do thymus-recirculating Foxp3⁺ Tregs come from, and do they return there?

How does the thymus foster the generation of multiple Treg precursors, and does this relate to any functional heterogeneity that may be present within thymus-derived Tregs?

Which aspects of medulla development and function should be targeted to manipulate thymic tolerance mechanisms?

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